REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE 30 May 1997	3. REPORT TYPE AND DATES COVERED Technical 6/1/96-5/31/97

4. TITLE AND SUBTITLE
Vibrational Sum Frequency Generation Studies of Saturated Glycerophospholipids: Effects of Temperature on Molecular Ordering at an Oil/Water Interface

5. FUNDING NUMBERS
N00014-89-J-1261

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8. PERFORMING ORGANIZATION REPORT NUMBER

ONR Technical Report #19

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Arlington, VA 22217-5000

10. SPONSORING/MONITORING AGENCY

11. SUPPLEMENTARY NOTES

In Press, J. Undergrad. Res.

12A. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release: distribution unlimited

12B. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Please see attached page

19970626 035

14. SUBJECT TERMS Saturated Glycerophospholipids; Oil/Water Interface	15. NUMBER OF PAGES 17
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT

DTIC QUALITY INSPECTED 1

OFFICE OF NAVAL RESEARCH

Grant N00014-89-J-1261

R&T Code 4131038

ONR Technical Report #19

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June 1997

Vibrational Sum Frequency Generation Studies of Saturated Glycerophospholipids: Effects of Temperature on Molecular Ordering at an Oil/Water Interface

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Abstract

Vibrational sum frequency spectra of 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) at the deuterium oxide-carbon tetrachloride interface were acquired over temperatures ranging from 10°C to 35°C. Temperature studies on DLPC ($T_c = -1$°C) were performed to determine the effects of thermal motion of solvent molecules on interfacial ordering, while DMPC ($T_c = 23$°C) temperature studies were carried out to elucidate relationships between the phase of bulk phospholipid bilayers, the degree of interfacial coverage, and molecular ordering. Results for DLPC suggested that thermal solvent motion does not significantly influence the degree of hydrocarbon chain ordering at the interface over the temperature region studied. This study also found that, in general, the ordering of DMPC at the interface increases as the temperature of the medium is increased to and beyond the phase transition temperature, T_c. The interfacial ordering of DMPC is discussed with respect to bilayer phase transitions in the bulk.

I. Introduction

Phospholipids are surface-active molecules which constitute the major component of biological membranes. Their prevalence in living organisms has motivated many studies on their properties in aqueous solution and at the air-water interface.[1-5] Very few studies, however, have

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provided molecular-level information on the behavior of phospholipids at liquid-liquid interfaces.[6] This investigation focuses on the relationship between molecular ordering, temperature, and interfacial coverage for two saturated glycerophospholipids, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), at the deuterium oxide-carbon tetrachloride interface. Such molecular-level information was acquired using the method of total internal reflection vibrational sum frequency generation (TIR-VSFG) to probe the CH-stretching region of the spectrum. This nonlinear optical method provides a selective probe of the molecules residing at the interface.

In aqueous solution, phospholipids form bilayer structures called vesicles. In these structures, the zwitterionic head groups are exposed to the polar solvent, while the hydrocarbon chains associate with each other by van der Waals forces. The tightness of chain packing and, in turn, the strength of the intermolecular forces within the vesicles depends upon the temperature of the medium. Phospholipid bilayers undergo an order-disorder phase transition at a characteristic phase transition temperature (T_c). Below T_c, the bilayer is in the gel phase. This phase is characterized by tightly-packed, well-ordered hydrocarbon chains. Once the temperature of the medium is raised above T_c, the bilayer melts into a liquid-crystalline phase in which the hydrocarbon chains are more fluid-like and less tightly-packed than they are in the gel. Intermolecular forces between the chains in the well-ordered gel phase are stronger than in the more disordered liquid-crystalline phase. Since it is the vesicles that supply phospholipids to the interfacial monolayer, an increase in temperature above T_c may result in more vesicles breaking apart to deposit molecules at the interface.

The objectives of this study involved determining the effects of temperature *and* interfacial concentration on molecular ordering at the boundary between two liquids. First, the effects of temperature on molecular ordering at the interface were examined using DLPC. Since the phase transition temperature of DLPC is $T_c = -1°C$, the bilayers in the bulk will be in the liquid-crystalline phase under reasonable experimental conditions (i.e., as long as the solvents are in their liquid phases). Therefore, the phase of the bilayer is fixed and only thermal effects from the solvents

influence the degree of ordering at the interface. After determining the effects of temperature on molecular ordering, relationships between order and interfacial concentration were investigated. DMPC, with its phase transition temperature at $T_c = 23°C$, was used to study this relationship. Since T_c of DMPC is near ambient temperature, the system afforded opportunities to conduct experiments well above and below the transition temperature. The interfacial concentration depends upon temperature due to the changes in the phase of the bilayers in solution. As previously stated, the forces between the hydrocarbon chains in the liquid-crystalline phase are expected to be weaker than in the gel phase and thus liquid-crystalline bilayers break apart more easily to form monolayers at the interface. Therefore, a change in the phase of the phospholipid bilayers in the bulk ought to alter the concentration of phospholipids at the interface. The ordering of interfacial phospholipids was monitored as the solution temperature was changed, and results were examined in light of the concentration of molecules at the interface and the relative strength of the intermolecular forces within the vesicle bilayers in the bulk.

II. Background

The method of total internal reflection vibrational sum frequency generation (TIR-VSFG) was employed in this study to obtain vibrational spectra of phospholipid hydrocarbon chains at the deuterium oxide-carbon tetrachloride interface.[7,8] Sum frequency generation is a second-order, nonlinear optical process which is inherently surface-specific.[9-11] In this spectroscopic method, coherent optical fields of two frequencies impinge on an interface, and a third field whose frequency is the sum of the incident frequencies is detected. Selection rules dictate that the sum frequency is only generated in noncentrosymmetric media. Therefore, only the interface is probed, while the inversion symmetry found in the bulk phases prevents them from contributing to the sum frequency spectra.

In VSFG one of the incident beams is visible light of fixed frequency, while the other is tunable, infrared radiation. The intensity of the resultant sum frequency signal is proportional to

the intensities of both incident fields and to the square of the second-order nonlinear susceptibility, $\chi^{(2)}$:

$$I_{SF} = \left| \chi^{(2)} \right|^2 I_{IR} I_{VIS} \tag{1}$$

$\chi^{(2)}$ consists of resonant and nonresonant components. The nonresonant component is relatively small and constant over the region of interest. The resonant component of $\chi^{(2)}$ can be represented by the following proportionality:

$$(\chi_{res}^{(2)})_{lmn} \propto \frac{N A_n M_{lm}}{\omega_{IR} - \omega_v + i\Gamma_v} \tag{2}$$

where N is the number of molecules adsorbed at the interface, A_n and M_{lm} are the infrared and Raman transition moments, respectively, ω_{IR} is the frequency of the tunable infrared radiation, ω_v is the frequency of a particular vibrational mode, and Γ_v is a damping constant equal to the linewidth. As the frequency of ω_{IR} approaches the frequency of a sum frequency-active vibrational transition, a resonant enhancement in the sum frequency occurs. This enhancement produces a vibrational spectrum of molecules residing at the interface.

In order to achieve the sensitivity required for the study of a liquid-liquid interface, a total internal reflection geometry is employed in the experimental apparatus. The incident beams are directed onto the interface through the higher index of refraction medium at their critical angles. This geometry creates an evanescent wave at the interface which results in an amplification of the local field intensity and the sum frequency signal (equation (1)). Operation in this configuration increases sensitivity several orders of magnitude above that attainable using a traditional external reflection geometry.

III. Experimental

The phospholipids examined in this study belong to a family of saturated, symmetric dialkyl phosphocholines. 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC, > 99%) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, > 99%) were purchased from Avanti Polar Lipids, Inc. and used without further purification. Deuterium oxide (Aldrich, > 99.9 atom % D) was employed as a solvent for the phospholipids and was buffered to pH 7.0 with sodium phosphate. Phospholipid solutions were sonicated above their phase transition temperatures for approximately 30 minutes, a process which resulted in the creation of unilamellar vesicles (diameter < 250 nm). Carbon tetrachloride (J. T. Baker, reagent grade) was used as a subphase to create the liquid-liquid interface. Phospholipid concentrations for the spectral study were chosen based on previous interfacial tension measurements which indicated the concentrations at which monolayers of the phospholipids are formed.[6] A DLPC concentration of 8.2 μM was used to form a monolayer of molecules. The DMPC concentration was 9.0 μM, which coincided with the initial formation of a monolayer above T_c. Interfacial pressure measurements were carried out using water (NANOpure, 17.8 Mohm-cm), similarly buffered to pH 7.0, instead of deuterium oxide. The Wilhelmy plate method was used to acquire interfacial pressure data. Interfacial pressure measurements were acquired using a platinum plate and a microbalance apparatus.

The experimental setup consisted of a laser system pumped by a Nd:YAG laser (Spectra-Physics, 1064 nm, 12 ns pulse width). The fundamental was split into two portions: one fraction was sent through a KDP doubling crystal to supply the fixed-frequency, visible (532 nm) radiation, while the remainder was used to pump a lithium niobate crystal in an optical parametric oscillator (OPO). The OPO generated tunable infrared radiation in the CH-stretching region (2750 to 3000 cm^{-1}). Polarization conditions were $S_{sum}S_{vis}P_{ir}$ which selected vibrational modes with transition moments oriented perpendicular to the interface. The beams were directed into a quartz sample cell where they converged at the liquid-liquid interface. The temperature of the cell was controlled by circulating a water/ethanol mixture through glass channels which passed through the

aqueous phase. In the DMPC studies, the temperature of the neat interface was cooled to 10°C before addition of the phospholipids. Higher temperature spectra were then acquired by sequentially increasing the temperature of the aqueous phase. DLPC temperature-controlled spectra were not acquired in this order since the bilayer phase over the entire experimental temperature range was liquid-crystalline. The sum frequency signal was detected using a PMT and gated electronics, and data acquisition was controlled by personal computer. Spectra Calc and Igor Pro computer programs were utilized to correct for infrared power fluctuations and to fit lineshapes.

IV. Results and Discussion

Spectra of DLPC at monolayer coverage were acquired over the temperature region of 10°C to 30°C in approximately 5°C increments. A sample VSFG spectrum of DLPC is shown in Figure 1. Strong signals are observed from the methyl and methylene symmetric stretching modes in this region. Spectral assignments are provided in Table I. Spectra were acquired using $S_{sum}S_{vis}P_{ir}$ polarization which selected transition moments perpendicular to the interface.

Results from fits to the spectral data were used to determine the ordering of molecules at the interface. The degree of order was determined from the ratio of the intensity of the methyl symmetric stretch to that of the methylene symmetric stretch.

$$\text{intensity ratio} = \frac{I_{CH_3ss}}{I_{CH_2ss}} \tag{3}$$

A higher ratio corresponds to a higher degree of order, for if the chains are in a fully-extended, all-*trans* conformation, the transition dipoles of the methylene symmetric stretch will cancel and this transition will contribute little to the VSFG spectrum. Once gauche deformations are introduced

into the hydrocarbon chains, the methylene symmetric stretch transition dipoles no longer undergo complete cancellation and a higher intensity ratio will be observed (for the same methyl symmetric stretch intensity). The relationship between methylene symmetric stretch intensity and molecular ordering can also be viewed in light of group theory. In an all-*trans* conformation, the environment of the methylene symmetric stretch is locally centrosymmetric. The possession of inversion symmetry prevents the transition from being both infrared- and Raman-active. Hence, one of these transition moments is zero and the mode becomes sum frequency inactive (equation (2)). Note that the terminal methyl groups of the molecules in the monolayer are not in a centrosymmetric environment for either the well-ordered or the disordered case, and therefore the methyl symmetric stretch is expected to contribute to all spectra.

Methyl-to-methylene symmetric stretch intensity ratios were determined for each spectrum. A plot of intensity ratio vs. temperature for DLPC is given in Figure 2. The average of all intensity ratios was 2.7(3), where the number in parentheses indicates the standard deviation. This ratio is in close agreement with those found in previous studies of DLPC at ambient temperature (average ratio = 2.8(1)).[6] Spectral data show that the intensity ratio does not change significantly over the temperature range probed to within experimental error. This trend implies that thermal motion of the solvent does not noticeably change the degree of ordering of phospholipid molecules at the interface over this temperature region.

DMPC spectra were acquired over the temperature range from 10°C to 35°C. Spectra of 9.0 μM DMPC at 18°C and 25°C are provided in Figure 3. A plot of intensity ratio vs. temperature appears in Figure 4. Additionally, interfacial pressure measurements were acquired at the same aqueous DMPC concentration from 10°C to 30°C. These data are shown in Figure 5.

There are several notable features in the DMPC data. First, even when it is most ordered, DMPC (maximum intensity ratio = 1.8(1)) is significantly less ordered at the interface than DLPC (average intensity ratio = 2.7(3)). This result presumably arises from the difference in chain lengths of the two phospholipids. With its longer chains, DMPC may be more susceptible to gauche defects than DLPC.

DMPC spectral data also show a general increase in order (larger methyl-to-methylene intensity ratio) at temperatures above the phase transition temperature. Such a result is consistent with the phase behavior of vesicles in the bulk. Below T_c, the intermolecular forces within the bilayer are stronger. Thus, fewer vesicles will break apart to deposit phospholipids at the interface. The expanded monolayer will show a lower degree of order due to the greater conformational mobility of the hydrocarbon chains. Interfacial pressure measurements confirm that the interfacial concentration is lower for $T < T_c$ relative to $T > T_c$, as a higher interfacial pressure correlates to a greater coverage at the interface. Above T_c, the vesicles can break apart more easily to supply the interface with a tightly-packed, more ordered phospholipid monolayer.

Two additional aspects of the DMPC data are of note. First, the highest intensity ratio (i.e., the most ordered monolayer) is observed at 22°C, which is below the phase transition temperature. Additionally, the interfacial pressure at 22°C is comparable to those above T_c. It then appears that greater ordering at this temperature arises from an interfacial coverage similar to that at temperatures above T_c. Raman spectroscopic studies of unilamellar DMPC vesicles in aqueous solution have shown that the gel to liquid-crystalline phase transition actually occurs over a temperature range spanning 21°C to 25°C.[3] The increased intensity ratio and interfacial pressure at 22°C are then reasonable given that the bilayers in the bulk are undergoing the phase transition in this temperature region, weakening the forces within the bilayer. Second, an increased intensity ratio similar to those at and above 22°C is observed at 14°C. This high intensity ratio may be anomalous. The interfacial pressure at this temperature is the lowest of all the measurements, suggesting that the increased intensity ratio is not a result of greater coverage at the interface. This temperature lies close to a 13.5(2)°C "pretransition" phase transition observed in differential scanning calorimetry experiments.[2] The pretransition corresponds to a change in the packing geometry of the headgroups in the bilayer. The ordering of the monolayer at 14°C may be influenced by this pretransition. Further experiments are necessary to verify the reproducibility of the ratios and interfacial pressures observed in the experimental data. Data acquired in these

additional experiments will help determine whether the data at 14°C is anomalous, or whether the large ratio provides some evidence of the pretransition.

V. Conclusions

The technique of total internal reflection vibrational sum frequency generation has provided a means of observing the degree of molecular ordering of phospholipids at a liquid-liquid interface. Temperature-controlled DLPC studies have suggested that thermal motion of the liquids has little effect on the relative order of the alkyl chains of this phospholipid at the interface at the temperatures probed. For DMPC, which has a transition temperature near room temperature, there is a clear difference in how the phospholipid orders above and below T_c. Below T_c less order is observed. This is indicative of the lesser concentration of phospholipids at the interface. Above T_c the relative ordering of the phospholipid hydrocarbon chains is generally higher. We attribute these differences to the bulk phase behavior of the phospholipids where they exist in gel phase at low temperatures and a liquid-crystalline phase at the higher temperatures. A heightened intensity ratio and interfacial pressure at 22°C can be accounted for by a broadened gel to liquid-crystalline phase transition region. An increased ratio at 14°C suggests a more ordered monolayer at this temperature, but interfacial tension measurements do not show a corresponding increase in interfacial coverage. Further experimentation is necessary to determine whether the nearby pretransition at 13.5(2)°C is influencing the ordering of the monolayer at 14°C.

VI. Acknowledgments

VII. References

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Figure and Table Captions

Figure 1. DLPC vibrational sum frequency generation spectrum at 25.5°C

Figure 2. Methyl-to-methylene symmetric stretch intensity ratio vs. temperature for DLPC. A representative error bar is included.

Figure 3. VSFG spectra of DMPC at 18.1°C and 25.1°C.

Figure 4. Methyl-to-methylene symmetric stretch intensity ratio vs. temperature for DMPC. Representative error bars are shown above and below T_c.

Figure 5. Surface pressure vs. temperature for DMPC. Representative error bars are shown above and below T_c.

Table I. Spectral assignments.

Figure 1

DLPC VSFG Spectrum: 25.5°C
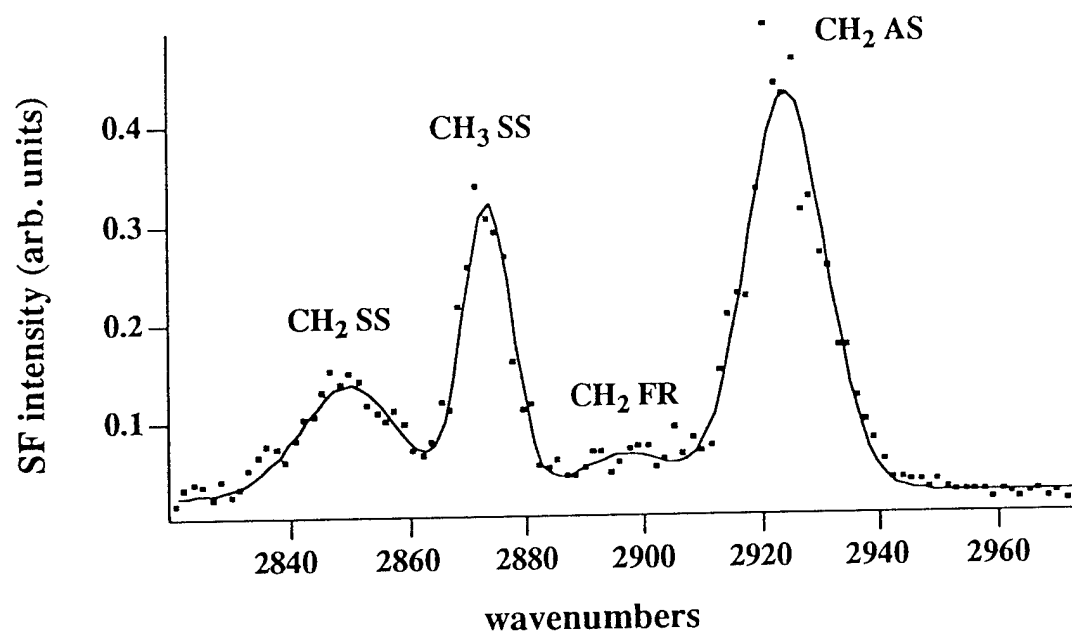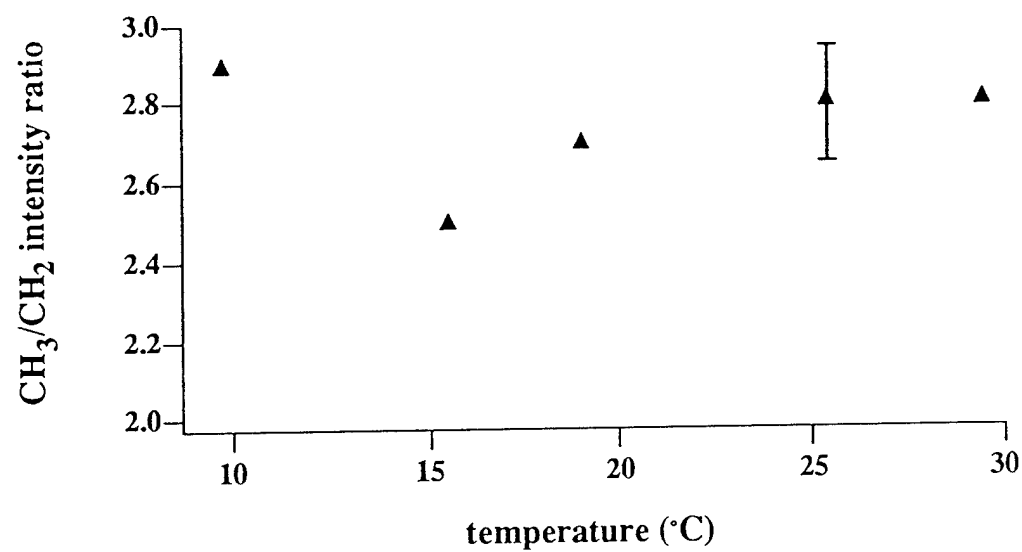
Figure 2

Intensity Ratio vs. Temperature

Figure 3

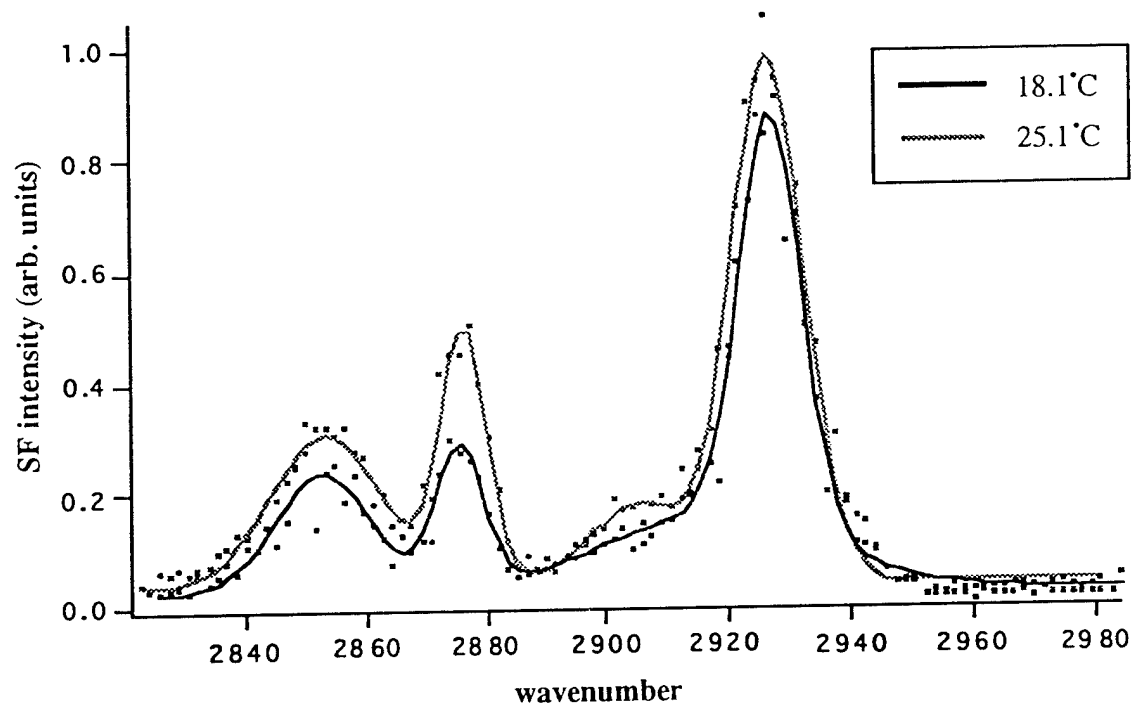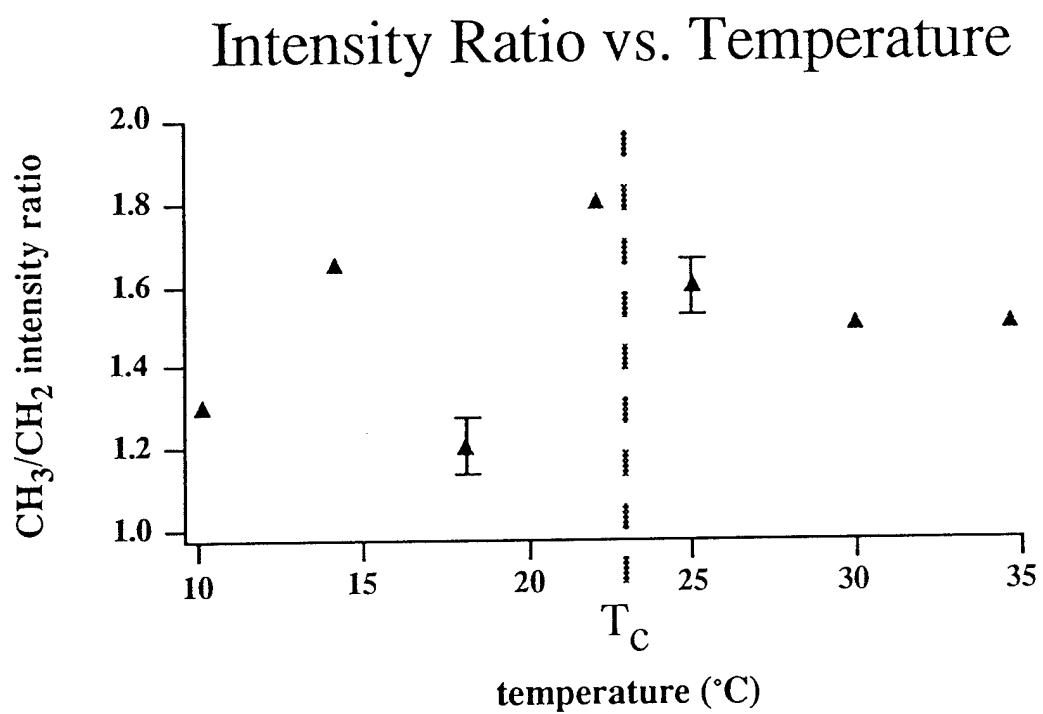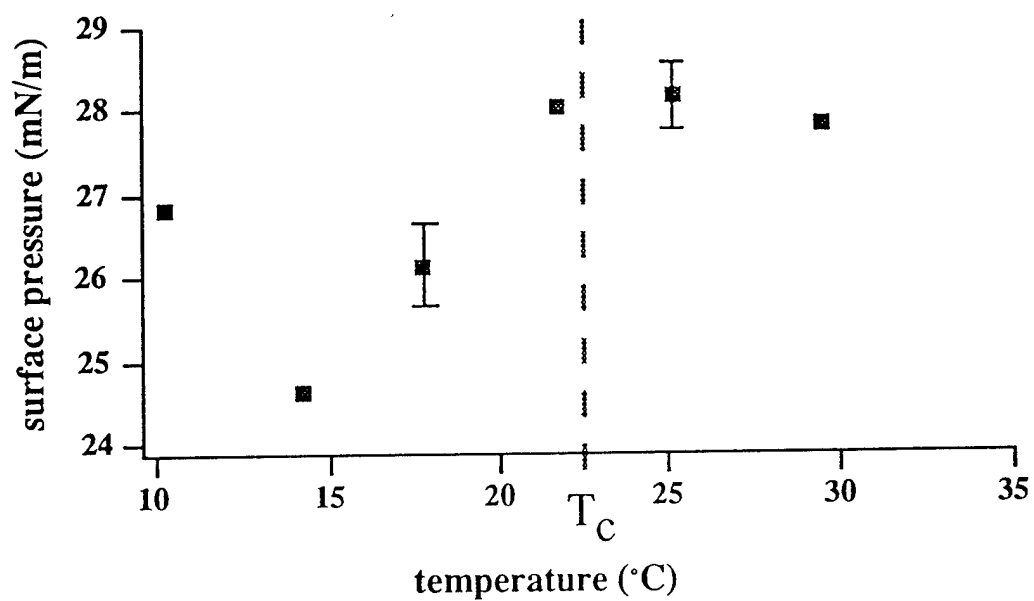DMPC VSFG Spectra: 18.1°C and 25.1°C

Figure 4

Intensity Ratio vs. Temperature

Figure 5

Surface Pressure vs. Temperature

Table I

Transition	Abbreviation	Position (cm^{-1})
Methylene symmetric stretch	CH_2 SS	2850 cm^{-1}
Methyl symmetric stretch	CH_3 SS	2872 cm^{-1}
Methylene Fermi resonance	CH_2 FR	2885-2915 cm^{-1}
Methylene antisymmetric stretch	CH_2 AS	2925 cm^{-1}